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Introduction of Macromolecules into Cultured Mammalian Cells by Osmotic Lysis of Pinocytic Vesicles

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Summary

We have developed a new procedure for introducing macromolecules into cultured mammalian cells based on osmotic lysis of pinocytic vesicles. Cells are first incubated in culture medium containing 0.5 M sucrose, 10% polyethylene glycol 1000 and the macromolecule to be transferred. Cells are then placed in medium diluted with 0.66 parts water. Most pinocytic vesicles formed in the presence of sucrose burst in hypotonic medium, thereby releasing the enclosed macromolecule. L929 cells remain fully viable after a single hypertonic sucrose treatment, and a majority survives four successive rounds of osmotic lysis. This procedure, termed osmotic lysis of pinosomes, has been used to transfer substantial amounts of horseradish peroxidase, antiricin antibodies and dextran 70,000 into the cytosol of L929 cells. Direct comparison of the degree of ricin resistance conferred by transfer of antiricin antibodies revealed pinosome lysis to be equal, if not superior, to injection mediated by red blood cells.

Introduction

Introduction of macromolecules into living cells is a powerful experimental approach, and several procedures have been developed for this purpose. The most versatile of these is direct microneedle injection (Diacumakos et al., 1970; Graessmann et al., 1974). Large amounts of macromolecules of almost any size can be injected into either the nucleus or cytoplasm of cultured mammalian cells. However, even by the most skilled hands, relatively few cells can be injected in a reasonable period of time. Therefore, two alternative methods have been developed that allow macromolecules to be transferred to large numbers of cells.

Fusion of artificial lipid vesicles (liposomes) with cultured cells can be used to inject macromolecules (Poste et al., 1976). Liposomes are attractive vehicles, since there are few constraints on the size of the macromolecules that can be encapsulated. Unfortunately, this advantage is offset by the small internal volumes and relatively poor fusability of unilamellar lipid vesicles. Moreover, results from liposome transfer experiments are sometimes equivocal due to endocytosis of the small lipid vesicles (see Pagano and Weinstein, 1978 for review).

The other method uses red blood cells as injection vehicles. In this procedure, macromolecules are introduced into red cells during hypotonic hemolysis, and the resealed red cells are fused to cultured cells by using Sendai virus (Furusawa et al., 1974; Loyter et al., 1975; Schlegel and Rechsteiner, 1975). Although this technique has proved useful, only relatively small macromolecules enter red blood cells during hemolysis. Proteins larger than 300,000 daltons (300 kd) are, therefore, not easily transferred, and the method does not work well with nucleic acids.

We report development of a third procedure that allows the large-scale transfer of macromolecules into the cytosol of cultured cells. L929 cells are exposed to medium containing 0.5 M sucrose, 10% polyethylene glycol 1000 and the macromolecule to be injected. Pinocytic vesicles form in this medium, and because of their increased internal osmotic pressure, they break when the cells are placed in hypotonic culture medium. These manipulations do not alter the viability of L929 cells, and this procedure has been used to introduce substantial amounts of horseradish peroxidase, fluoresceinated dextran 70,000 and antiricin antibodies into the cytoplasm of these cells.

Results

Uptake of Horseradish Peroxidase in the Presence of Sucrose

Transferring macromolecules by osmotic lysis of pinocytic vesicles requires that cells continue pinocytosis in hypertonic medium. The uptake of horseradish peroxidase (HRP) was used to measure pinocytosis, since it has been shown to be a good marker of fluid phase pinocytosis in L929 cells (Steinman et al., 1974, 1976). HRP uptake by L929 cells was inhibited more than twofold in medium containing 0.5 M sucrose alone (data not shown). However, in medium containing both 0.5 M sucrose and 10% polyethylene glycol 1000 (PEG 1000), the rate of HRP uptake was more than double that observed in L929 cells in unsupplemented medium (Figure 1). Thus continued pinocytosis in hypertonic medium, a major requirement for injection based on osmotic lysis of pinosomes, was obtained.

The Intracellular Distribution of HRP

When L929 cells are placed in culture medium containing 0.5 M sucrose, they lose water and shrink; they regain normal volumes after return to culture medium. Pinocytic vesicles formed prior to hypertonic shock presumably behave similarly to whole-cell cytoplasm and do not burst upon rehydration. In contrast, those pinocytic vesicles formed in medium containing 0.5 M sucrose should have an internal osmotic pressure of 0.8 osmolar, and these vesicles should swell and break upon rehydration of the cytosol.

We tested this prediction as follows. Confluent monolayers of L929 cells were incubated for 10 min in medium containing 4 mg/ml HRP (control cells) or

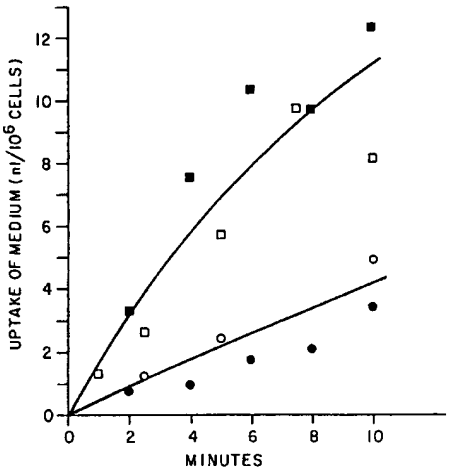

Figure 1. Effects of Sucrose and PEG 1000 on Rates of Fluid Phase Pinocytosis in L929 Cells

Confluent monolayers of L929 cells were exposed for the times indicated to HEPES-buffered F12 medium containing 2 mg/ml HRP in the presence (squares) or absence (circles) of 0.5 M sucrose and 10% PEG 1000. The cells were then rinsed thoroughly in unmodified culture medium and extracted by freeze-thawing, and the amount of HRP internalized was used to calculate fluid uptake. Each entry is the average from two culture dishes; open and closed symbols represent separate experiments.

Table 1. Distribution of Horseradish Peroxidase after Osmotic Lysis of Pinosomes

	Control		Osmotic Lysis Procedure	
	Total Enzyme		Total Enzyme	
Fraction	(ng)	%	(ng)	%
Homogenate	355	100	600	100
Postnuclear supernatant	280	79	702	117
Nuclear pellet	81	23	42	7
Mitochondria lysosomes	170	48	126	21
Postlysosomal supernatant	74	21	504	84

Confluent monolayers of L929 cells on 100 mm petri dishes were incubated in HEPES-buffered F12 medium containing 4 mg/ml HRP or in osmotic lysis medium containing 4 mg/ml HRP for 10 min prior to 2 min of hypotonic treatment (osmotic lysis procedure) or rinsing with unmodified F12 medium (control). After thorough rinsing, cells from two petri dishes receiving each treatment were fractionated separately to ensure that the fractionation procedure was reproducible. The distribution of HRP was determined in the various fractions; each columnar entry is the mean from two fractionations that differed less than 20%. The percentage of HRP distribution was obtained by comparing the HRP in each fraction to the amount of HRP in the homogenate, so the values do not necessarily sum to 100%. Control dishes contained an average of 9.6×10^6 L929 cells; osmotic lysis dishes contained an average of 9.3×10^6 L929 cells.

in medium supplemented with 0.5 M sucrose–10% PEG 1000 and 4 mg/ml HRP. The control cells were then rinsed in normal medium, whereas cells exposed to HRP in sucrose were incubated in hypotonic F12 medium (six parts F12 to four parts water) for 2 min prior to rinsing with normal medium. Both sets of cells were fractionated after thorough rinsing. The results in Table 1 show that over 80% of the HRP internalized in the presence of sucrose was found in the postlysosomal supernatant as compared to only 20% in control cells. Further sedimentation of the postlysosomal supernatant at 100,000 × g for 2 hr did not reduce the amount of soluble HRP.

The above results indicate that HRP escaped pinocytic vesicles upon hypotonic treatment. We could not, however, exclude the possibility that pinocytic vesicles formed in the presence of sucrose ruptured during fractionation. For this reason, we examined the distribution of HRP by cytochemistry. Figures 2A and 2B show the distribution of HRP in L929 cells that were exposed to HRP in medium or in medium containing 0.5 M sucrose, rinsed in the same medium and fixed with 2% glutaraldehyde in phosphate-buffered saline. In both cases, the enzyme was clearly confined to vesicles. In contrast, if cells that incorporated HRP in medium containing 0.5 M sucrose and 10% PEG 1000 were placed in hypotonic F12 medium (six parts F12 to four parts water) prior to glutaraldehyde fixation, then the enzyme was found dispersed in the cytoplasm (Figure 2C) and occasionally concentrated within nuclei (Figure 2D). Comparable results were obtained when the intracellular distribution of the water-soluble fluorescent dye, Lucifer yellow, was determined by fluorescence microscopy (not shown).

Conditions for Release of HRP from Pinocytic Vesicles

Histochemical staining was used to determine requirements for HRP release from pinocytic vesicles because of the ease with which the procedure revealed the intracellular distribution of HRP. These studies, some of which are reported below, resulted in a standard protocol for hypotonic lysis of pinocytic vesicles. The macromolecule to be transferred is dissolved in F12 medium lacking sodium bicarbonate, buffered at pH 6.8 with 10 mM HEPES and containing 5% fetal calf serum. The medium is brought to 0.5 M sucrose by addition of 1 M sucrose in HEPES-buffered F12 and to 10% PEG 1000 by addition of 50% PEG 1000 dissolved in regular F12 medium. Cells are exposed to this final medium for 10 min, rapidly rinsed with hypotonic, HEPES-buffered F12 medium (six parts F12 to four parts water) and incubated in the same medium for 2 min. The cells can then be rinsed with normal culture medium and treated as desired. The phrase "osmotic lysis of pinosomes" describes the above procedure, which is shown schematically in Figure 3.

PEG 1000 at a concentration of 5% or greater was

Figure 2. Histochemical Localization of HRP before and after Osmotic Lysis of Pinocytic Vesicles

(A) Cells incubated for 10 min in HEPES-buffered F12 medium containing 10 mg/ml HRP prior to fixation and staining as described in Experimental Procedures. (B) L929 cells incubated for 10 min in medium containing 0.5 M sucrose, 10% PEG 1000 and 10 mg/ml HRP and rinsed with medium containing 0.5 M sucrose prior to fixation and staining. (C and D) Cells exposed to the same medium as the cells in (B), but placed in hypotonic F12 (six parts F12 medium to four parts water) for 2 min and rinsed in unmodified F12 medium prior to fixation and histochemical staining for HRP.

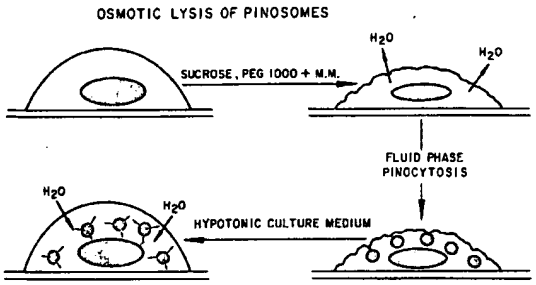

Figure 3. Schematic Representation of the Osmotic Lysis of Pinosomes

M.M.: macromolecules.

required during incubation in sucrose or HRP was not released from the pinocytic vesicles. This was shown by various combinations of serial incubation. Only when HRP, sucrose and PEG 1000 were together during incubation did HRP escape pinocytic vesicles upon exposure of treated cells to hypotonic F12. Dextran 10,000 at 5%, 20% or 45% would not substitute for 10% PEG 1000, suggesting that the requirement for PEG 1000 is not an osmotic one.

When L929 cells were exposed for 9 min to osmotic lysis medium containing 2 mg/ml HRP, rinsed with and then incubated in F12 medium containing only PEG 1000 and sucrose for 1 min and then subjected to hypotonic shock, histochemical staining showed intense HRP reaction product distributed throughout the cytoplasm. In contrast, when cells were incubated in F12 medium containing 10% PEG 1000 and 0.5 M sucrose for 9 min and incubated in osmotic lysis medium containing 2 mg/ml HRP for 30 sec prior to hypotonic shock, there was only faint cytoplasmic staining. Thus significant amounts of HRP do not enter cells from the medium upon addition of hypotonic F12 medium.

Comparison of Figures 2C and 2D shows that the intracellular distribution of HRP may vary following osmotic lysis of pinosomes. Although HRP appears confined to the cytoplasm in most cells, a minority (generally 5%–30%) displays intense nuclear labeling. In cells allowed to take up HRP in osmotic lysis medium for 3, 6, 12 or 18 min prior to hypotonic treatment, the proportion of cells showing nuclear labeling increased with longer incubation. Moreover, in cells subjected to multiple rounds of HRP uptake and lysis, nuclear labeling was as high as 80% after four rounds of uptake. These observations suggest that nuclear localization of HRP may result from osmotically induced changes in the permeability of the nuclear membrane. It should be noted that HRP may be found in the nucleus after injection mediated by red blood cells as well (unpublished observation).

Histochemistry was also used to determine whether HRP could be transferred by osmotic lysis of pinosomes formed during suspension of L929 cells. Freshly trypsinized cells were washed three times with 10 ml of F12 medium and incubated in a 37°C water bath at 10^7 cells per 100 μl in osmotic lysis medium containing 4 mg/ml HRP; control cells were incubated in unmodified medium containing 4 mg/ml HRP. After 10 min, cells incubated in sucrose–PEG 1000 were diluted with 10 ml of hypotonic F12 and collected by centrifugation 2 min later. Both sets of cells were then washed with F12 medium, plated onto coverslips and fixed with 2% glutaraldehyde 1 hr later. Subsequent histochemical staining showed that in cells which had taken up HRP in the presence of 0.5 M sucrose and 10% PEG 1000, the enzyme was dispersed throughout the cytoplasm. In control cells, HRP was confined to perinuclear vesicles presumed to be lysosomes. The ability of L929 cells to incorporate HRP from suspension should permit conservation of precious macromolecules, since the higher ratio of cell volume to medium volume in a cellular pellet is more favorable for uptake than exposure of cells in monolayer.

Osmotic Lysis of Pinosomes Does Not Release Lysosomal Enzymes

Steinman et al. (1976) observed that HRP began to accumulate in L929 lysosomes 5 min after addition to the medium. We observed release of HRP upon osmotic lysis of cells exposed to HRP for only 2 min, so it is unlikely that fusion of pinosomes and lysosomes is required for HRP release to the cytosol. However, since cells are normally incubated in sucrose for 10 min prior to hypotonic shock, we asked whether the osmotic lysis procedure caused release of lysosomal enzymes. L929 cells were incubated in standard osmotic lysis medium for 0, 5, 10, 20 or 30 min prior to hypotonic shock. Cells from each treatment were then fractionated, and we observed no significant differences in the total amounts of acid phosphatase recovered or in the proportion of the enzyme in the postlysosomal supernatant fractions (data not shown). These results suggest that sucrose-laden, pinocytic vesicles do not readily fuse with lysosomes, or if they do, lysosomal enzymes are not released upon osmotic lysis.

Cell Viability after Lysis of Pinosomes

If cells died after osmotic lysis of pinocytic vesicles, the procedure would have limited usefulness. Fortunately, as shown by the following experiment, they do not. Confluent monolayers of L929 cells growing on 18 mm circular coverslips were exposed to HEPES-buffered F12 medium containing 0.5 M sucrose and 10% PEG 1000 for 5, 10 or 20 min prior to hypotonic shock. Fifteen minutes later, cells were removed from each coverslip by trypsinization, washed in F12 medium and counted, and 10,000 cells were plated into each of two 25 cm^2 T flasks. The plating efficiencies presented in Table 2 were calculated from the number

Table 2. Cell Viability and Growth Rate after Osmotic Lysis of Pinocytic Vesicles

Minutes in Sucrose	Hypotonic Shock[a]	Plating Efficiency	Number of Colonies with the Indicated Cells per Colony 72 hr after Plating				
			1	2	3–4	5–8	>8
0	–	0.43	4	21	75	19	5
5	+	0.42	ND	ND	ND	ND	ND
10	+	0.50	6	35	52	24	4
20	+	0.12	ND	ND	ND	ND	ND

[a] Column denotes whether cells were exposed to 60% HEPES-buffered F12 for 2 min.
ND: not determined.

of single cells and cell pairs observed in 20 random microscope fields on the following day. Although 20 min of exposure of L929 cells to medium containing 0.5 M sucrose resulted in a fourfold decrease in viability, 5 or 10 min exposures had no effect. Moreover, when colony size distributions were measured 72 hr after plating, no difference was observed between cells exposed to sucrose for 10 min prior to hypotonic shock and control cells (Table 2). Thus a single round of osmotic lysis diminishes neither the viability nor the growth rate of L929 cells.

Multiple Rounds of Osmotic Lysis

Whether cells can survive multiple cycles of osmotic lysis of pinosomes is a question of interest to those who might wish to transfer large quantities of macromolecules into cells. The results in Figure 4 demonstrate that roughly equivalent amounts of HRP were internalized during each of four successive rounds of osmotic lysis separated by only 5 min of culture in normal medium. Trypan blue dye exclusion assays on L929 cells on parallel coverslips revealed that virtually all cells survived three rounds of osmotic lysis, but from 20% to 40% cell death was observed after the fourth round.

Immunoglobulin G Molecules and Dextran 70,000 Also Escape from Pinocytic Vesicles upon Osmotic Shock

The combination of fractionation and histochemical studies reported above leaves little doubt that most internalized HRP molecules are released to the cytosol upon hypotonic shock of sucrose-laden pinocytic vesicles. HRP is a relatively small protein (40 kd), and the versatility of the osmotic lysis procedure depends upon whether larger macromolecules also escape pinocytic vesicles. Accordingly, we used a functional assay for antiricin activity to test whether rabbit IgG (150 kd) was released.

KB cells exhibit markedly increased resistance to diphtheria toxin after receiving an injection of anti-diphtheria toxin antibodies (Yamaizumi et al., 1978). This observation suggested a functional assay for antibody transfer by osmotic lysis of pinosomes. Because mouse cells are not very sensitive to diphtheria

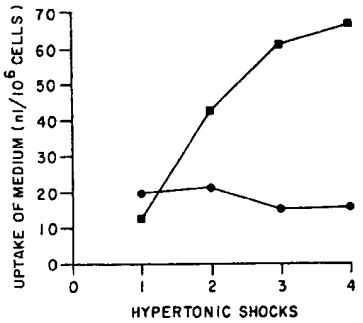

Figure 4. HRP Uptake during Multiple Cycles of Osmotic Lysis of Pinocytic Vesicles

Confluent monolayers of L929 cells in 60 mm petri dishes were subjected to multiple cycles of osmotic lysis in the presence of 2 mg/ml HRP. Two cultures were extracted after each round of lysis, and the internalized HRP was measured (■). In a separate experiment to control for possible HRP toxicity, cells were subjected to a single osmotic lysis in the presence of 2 mg/ml HRP after 0, 1, 2 or 3 prior cycles of osmotic lysis in 0.5 M sucrose–10% PEG 1000 alone (●). Each data point is the mean from two flasks. Both sets of data demonstrate that several rounds of osmotic lysis do not markedly inhibit HRP uptake on a subsequent round.

toxin, we measured antibody-mediated resistance to ricin. The data in Table 3 show that cells exposed to antiricin antibody under standard conditions for osmotic lysis of pinosomes are resistant to higher levels of ricin than cells exposed to antiricin antibody in unmodified culture medium or cells exposed to components of the hypertonic shock medium. Moreover, direct comparison of the transfer of antiricin antibodies by osmotic lysis of pinosomes and by the red cell fusion method shows that osmotic lysis confers comparable, if not superior, resistance to ricin toxicity (Table 4).

Cell fractionation was used to determine whether fluoresceinated dextran 70,000 was released upon osmotic lysis of pinosomes. L929 cells were exposed to a mixture of fluoresceinated dextran 70,000 and HRP for 10 min in the presence or absence of 0.5 M sucrose and 10% PEG 1000. Cells treated with sucrose–PEG 1000 were hypotonically shocked, and both sets of cells were fractionated after thorough rinsing. The data in Table 5 show that the subcellular distributions of HRP and dextran 70,000 are similar,

Table 3. Ricin Resistance of L929 Cells after Osmotic Lysis in the Presence of Antiricin IgG

Ricin (ng/ml)	Colonies per 20,000 Cells Plated				
	Control	Sucrose Plus PEG 1000	PEG 1000 Alone	Antiricin Plus PEG 1000	Antiricin Plus Sucrose Plus PEG 1000
0	10,370	10,250	10,750	11,375	11,550
10	0	0	0	0	9,275
50	0	0	0	0	3,850
250	0	0	0	0	16

Confluent monolayers of L929 cells growing on 18 mm circular coverslips were exposed to medium containing 0.5 M sucrose, 10% PEG 1000 and 10 mg/ml antiricin IgG or to various components of this mixture for 7 min. Cells exposed to the complete mixture were then incubated in hypotonic F12 medium for 2 min, whereas cells on other coverslips were rinsed in unmodified F12 medium. After 4 hr, cells were collected from each coverslip by trypsinization, washed and plated into numerous 25 cm^2 T flasks at 20,000 cells per flask. After 30 hr, cells were challenged with various concentrations of ricin for 36 hr. Cells were then fed every 2 days and fixed 5 days later. At high densities, colonies (defined as 16 or more cells) were determined by examining 20 random fields on an inverted microscope. At lower densities cells were stained with Giemsa blood stain and colonies were counted under a dissecting microscope. Each entry is the average from two flasks.

Table 4. Comparison of Ricin Resistance Conferred by the Transfer of Antiricin Antibodies by Osmotic Lysis and by the Red Cell Fusion Method

Ricin (ng per ml)	Colonies per 20,000 Cells Plated			
	Control	Mock-Injected	Injected with Antiricin	Osmotic Lysis of Pinosomes
0	8960	8580	7975	7865
10	0	0	1870	6765
50	0	0	248	3300
250	0	0	4	142
1250	0	0	0	0

Rabbit antiricin IgG at 10 mg/ml was loaded into red blood cells or dialyzed into osmotic lysis medium. By using standard procedures (Schlegel and Rechsteiner, 1978), L929 cells were then fused with IgG-loaded red blood cells (injected antiricin) or, because we have found that fusion of red blood cells and HeLa cells confers significant resistance to diphtheria toxin, L929 cells were fused to red blood cells loaded in the presence of 10 mM Tris (pH 7.4) alone (mock-injected). At the same time, L929 cells on 18 mm circular coverslips were subjected to standard osmotic lysis in the presence of 10 mg/ml antiricin (osmotic lysis) or received no treatment (control). Eighteen hours later, cells were collected by trypsinization and 20,000 cells from each treatment were plated into numerous 25 cm^2 T flasks. After an additional 12 hr, cells were challenged with various levels of ricin for 48 hr, cultured 6 more days and fixed, and colonies (≥16 cells) were determined as described in Table 3.

indicating that the two molecules were released in parallel after osmotic lysis. Since the molecular radius of dextran 70,000 is equivalent to that of a protein of 300–400 kd, osmotic lysis of pinosomes appears suitable for the transfer of large proteins, perhaps even macromolecular assemblies.

Discussion

Cells can internalize macromolecules either by fluid phase or by receptor-mediated endocytosis (Silverstein et al., 1977; Goldstein et al., 1979). Mosley et al. (1981) recently exploited receptor-mediated endocytosis of pyrene-labeled low density lipoprotein followed by photosensitization to kill cells with low density lipoprotein receptors. In the experiments described above, we obtained evidence that fluid phase endocytosis can be used to transfer macromolecules into the cytosol of cultured mammalian cells. This evidence includes changes in the intracellular distribution of HRP and fluoresceinated dextran 70,000 determined by cell fractionation (Tables 1 and 5), functional assays for the transfer of antiricin antibodies (Tables 3 and 4) and changes in the distribution of HRP determined histochemically (Figure 2).

We believe that these experiments together provide convincing evidence for osmotic lysis of pinosomes and release of their macromolecular contents. However, each experiment is not, by itself, necessarily compelling. For example, it could be argued that hypotonic shock labilizes vesicles, which rupture upon fractionation. On this hypothesis the data in Tables 1 and 5 would be fractionation artifacts.

Functional assay for antiricin activity provides better evidence for osmotic lysis of pinosomes. It is known that ricin intoxication results from inactivation of the 60S ribosomal subunit in the cytosol (Zamboni et al., 1981). In studies on diphtheria toxin, a molecule whose toxic action also occurs in the cytosol, Yamaizumi et al. (1978) presented evidence that injected antidiphtheria toxin antibodies protected cells by coupling the toxic fragment of diphtheria toxin within the cytosol, and by analogy, one might infer that antiricin antibodies act in the cytosol. This inference would be better justified if it were known that ricin entered the cytosol through the plasma membrane, as proposed some years ago for entry of diphtheria toxin (Boquet et al., 1976). In fact, recent studies suggest that diphtheria toxin may enter the cytosol from lysosomes (Draper and Simon, 1980; Sandvig and Olsnes, 1980). Thus it might be suggested that ricin resistance is provided by antibodies retained within lysosomes. The data in Table 3 provide evidence that this is not the case. L929 cells exposed to antiricin antibody in the absence of sucrose and hypotonic lysis were no

Table 5. Distribution of Horseradish Peroxidase and Fluoresceinated Dextran 70,000 after Osmotic Lysis of Pinosomes

	Control				Osmotic Lysis Procedure			
Fraction	FITC Dextran (ng)	HRP (ng)	% FITC Dextran	% HRP	FITC Dextran (ng)	HRP (ng)	% FITC Dextran	% HRP
Homogenate	640	257	100	100	1065	728	100	100
Postnuclear supernatant	330	126	52	49	990	701	93	96
Nuclear pellet	276	140	43	55	195	63	18	9
Mitochondria lysosomes	272	131	43	51	345	81	32	11
Postlysosomal supernatant	240	37	38	15	855	608	80	84

Confluent monolayers of L929 cells on 100 mm petri dishes were incubated in HEPES-buffered F12 media containing 2.5 mg/ml HRP and 5 mg/ml FITC (fluorescein isothiocyanate) dextran or in osmotic lysis medium containing 2.5 mg/ml HRP and 5 mg/ml FITC dextran for 10 min prior to 2 min of hypotonic treatment (osmotic lysis procedure) or rinsing with unmodified F12 medium (control). After thorough rinsing, cells from three petri dishes for each procedure were fractionated. The distribution of HRP and FITC dextran were determined in the various fractions. The approximate number of cells fractionated from the control dishes was 2.6×10^7 L929 cell; on the sucrose dishes there were 2.5×10^7 L929 cells. The percentage of HRP and FITC fluorescein distributions are calculated relative to the amounts present in the original homogenates. Recovery varies so the values do not necessarily sum to 100%. L929 cells contain virtually no endogenous HRP activity, and endogenous fluorescence is less than 15% of the fluorescence observed after uptake of FITC dextran.

more resistant than control cells to ricin. In one preliminary experiment, increased ricin resistance was observed in cells exposed to antiricin alone, but this resistance was significantly less than that observed in cells exposed to both antiricin antibodies and sucrose.

Moreover, the data in Table 4 show that injection by osmotic lysis of pinosomes or by the red cell fusion method confer approximately equal levels of ricin resistance upon L929 cells. The following calculation shows this to be expected. A red blood cell contains approximately 6×10^5 IgG molecules after loading in the presence of 10 mg/ml IgG under standard conditions (Schlegel and Rechsteiner, 1978). Since transfer is quantitative upon fusion of red blood cells and culture cells, each injected L929 cell should contain 6×10^5 IgG molecules. From the data in Figure 1 it can be calculated that in 10 min an L929 cell internalizes slightly more than 0.01 picoliters of medium containing sucrose and PEG 1000. A 10 mg/ml solution of IgG contains 4×10^{16} molecules per milliliter or 4×10^5 molecules per 0.01 picoliter. Thus the resistance conferred by osmotic lysis of pinosomes is very close to that expected on the basis of injection mediated by red blood cells.

Histochemical localization of HRP provides the best evidence that macromolecules can escape pinocytic vesicles upon osmotic lysis. The retention of virtually all HRP reaction products within vesicles in L929 cells fixed in 0.5 M sucrose (Figure 2B) eliminates the possibility that the enzyme escapes vesicles upon glutaraldehyde fixation. Moreover, when cells were incubated in medium containing HRP and sucrose, but lacking PEG 1000, the HRP reaction product was confined to vesicles even after hypotonic treatment. Thus there is no reason to suspect that the histochemical localization of HRP is in error.

The extensive studies by Steinman et al. (1974, 1976) on HRP uptake by L929 cells showed that the enzyme entered these cells in uncoated vesicles formed by fluid phase pinocytosis. The absence of receptors for HRP in this cell line makes it very likely that the enzyme is incorporated into L929 cells by the same pathway in medium containing sucrose and PEG 1000. Therefore, the technique reported here differs from several procedures in which molecules appear to enter culture cells directly through the plasma membrane.

Pardee and his colleagues have developed two procedures for introducing molecules into culture cells. In the first (Castellot et al., 1978), cells are cultured in medium made hypertonic by addition of NaCl. After 40–50 min, more than 90% of the cells stain with trypan blue, and they will incorporate normally nonpermeating, small molecules, such as nucleotides, into polymers. Cells show excellent viability upon reversal of the hypertonic shock. In the second procedure (Miller et al., 1978, 1979), cells are treated with low levels of lysolecithin. Under these conditions cells will take up proteins, such as RNAase, and carry out macromolecular syntheses, but they are inviable after treatment. Thus the latter technique is suitable only for relatively short-term studies.

Recently, Yarosh and Setlow (1981) described a procedure for introducing Micrococcus luteus endonuclease into hamster cells that was based on incubating cells in 50% PEG 6000 prior to adding enzyme. Although it is clear from their data that endonuclease entered the hamster cells, the pathway of entry remains unknown. Interestingly enough, one step in the procedure involves 30 min of incubation of cells in low levels of PEG 6000 and enzyme. Hence uptake may have involved pinocytosis rather than permeabilization of the plasma membrane. It is known, however, that the procedure of Yarosh and Setlow produces some loss of cell viability and cell–cell fusion.

The unimpaired viability of L929 cells following os-

motic lysis of pinosomes, documented in Table 2, is a major advantage of our technique. Excellent viability was observed even after three rapid rounds of osmotic lysis. Although a fourth round produced some cell killing, with proper temporal spacing, high viability after numerous rounds of osmotic lysis might be obtained. The excellent viability apparently results from the fact that the presumed membrane pores generated by osmotic lysis occur in sealed, isolated vesicles within the cytoplasm rather than in the plasma membrane itself.

If fluid phase pinocytosis is the mechanism by which L929 cells internalize molecules from medium containing sucrose and PEG 1000, then osmotic lysis of pinosomes may permit the transfer of very large molecules into the cytosol. Steinman et al. (1976) estimated the average diameter of L929 pinocytic vesicles at 0.2 μm, and the data in Table 5 show equivalent release of HRP (40 kd) and fluoresceinated dextran 70,000 (protein equivalent of 300 kd or more). The favorable combination of ample vesicle size and the apparent size-independent release of vesicle contents suggests that we may be able to transfer macromolecular assemblies, such as ribosomes, into cultured mammalian cells.

However, osmotic lysis of pinosomes has limitations. One was revealed when we attempted to determine whether ^{125}I-ferritin and HRP were released in parallel upon lysis of pinosomes. Whereas after 10 min of incubation in osmotic lysis medium, 10^7 L929 cells internalized 0.15 μl of medium by HRP assay, they "internalized" several μl based on ^{125}I-ferritin uptake. Subsequent fractionation showed most ^{125}I-ferritin in particulate fractions, presumably bound to the plasma membrane. Hence molecules that bind significantly to plasma membranes may present problems.

Another possible limitation is restriction to cells that exhibit high rates of fluid phase pinocytosis. Steinman et al. (1974) found that growing L929 cells, 3T3 cells, SV40-transformed 3T3 cells and calf embryo fibroblasts exhibited roughly equal rates of fluid phase pinocytosis. They also observed that the process was two to four fold faster in confluent L929 cells. Since we have used confluent L929 cells for virtually all studies reported above, one may not obtain as much transfer in other cell lines. Nevertheless, we know that the procedure works with both 3T3 and HeLa cells as well, and qualitative estimates from the histochemistry assay suggest that comparable amounts of HRP are internalized. Thus, despite these demonstrated or potential limitations, osmotic lysis of pinosomes should prove to be a useful technique. Compared to injection mediated by red blood cells, it is simple. Furthermore, it may allow transfer of very large macromolecules. We are currently asking whether it can be used to introduce cloned thymidine kinase genes into LM C/1D cells.

Experimental Procedures

Cell Culture

Cells of the heteroploid mouse cell line, L929, were provided by M. Capecchi and used in most experiments. D98/AH2 cells, a heteroploid human cell line, were obtained from the American Type Culture Collection; mouse 3T3-4E cells were provided by Dr. H. Coon, National Institutes of Health. Cells were grown in F12 medium supplemented with 4% calf serum and 1% fetal calf serum but lacking antibiotics. During experimental manipulation, penicillin and streptomycin were added to the culture medium to final concentrations of 100 units/ml and 50 μg/ml, respectively. Cells were routinely subcultured by trypsinization in 0.1% trypsin in Ca- and Mg-free saline. Cell numbers were determined with a hemacytometer.

Microinjection Mediated by Red Blood Cells

Red cells were loaded with IgG and fused to L929 cells according to the methods described by Schlegel and Rechsteiner (1978).

Determination of Pinocytic Rates

Rates of pinocytosis were measured by using horseradish peroxidase as the pinocytic marker (Steinman et al., 1974). L929 cells, grown to confluency on 60 mm petri dishes in bicarbonate-buffered F12 medium, were maintained in F12 medium, buffered with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) at pH 6.8 for up to 1 hr prior to measurements of pinocytosis. Cells were incubated in 2–5 mg/ml HRP in HEPES-buffered F12 (control) or in HEPES-buffered F12 supplemented with 0.5 M sucrose, 10% PEG 1000 and 2–5 mg/ml HRP. Uptake of HRP was terminated by rinsing with 5 ml HEPES-buffered F12 followed by eight 5 ml washes with HEPES-buffered F12 over 40 min. Cells were then rinsed twice with 5 ml of phosphate-buffered saline and lysed by freeze-thawing in 1 ml of 0.05 M sodium phosphate (pH 5.0). HRP activity was measured in the assay mixture described by Steinman et al. (1974), and the concentration of HRP in cell lysates was determined by comparison to a standard curve generated on the same day.

Cell Fractionation and Protein Determination

Cells were fractionated as described by Bigelow et al. (1981). Acid phosphatase was assayed by the method of Hall et al. (1978), and protein was determined by the method of Lowry et al. (1951).

Fluorescence Measurements

The concentration of FITC dextran 70,000 in various cellular fractions was measured on an Aminco-Bowman Spectrophotofluorometer (American Instrument). Excitation wavelength was 490 nm and fluorescence emission was measured at 520 nm.

In experiments where Lucifer yellow was used as the pinocytic marker, cells were grown on 18 mm circular glass coverslips. Following 5–10 min of incubation in 0.6% (w/v) Lucifer yellow in media or in media supplemented with 10% PEG 1000 and 0.5 M sucrose, both sets of cells were rinsed five times with F12 medium and then fixed as described by Stewart (1978). Coverslips were mounted on slides with glycerin and examined by epifluorescence through filters matched for the detection of fluorescein on a Zeiss Photomicroscope II. Under these conditions Lucifer yellow fluoresces green and background autofluorescence is yellowish-brown.

Histochemical Detection of HRP

Cells on coverslips were rinsed in medium and then phosphate-buffered saline (PBS) with or without 0.5 M sucrose depending upon the experiment, fixed for 1–10 min in 2% glutaraldehyde in PBS, rinsed thoroughly in PBS and stained by the diaminobenzidine method described by Steinman et al. (1974). Cells were counterstained with Giemsa and photographed on a Zeiss Photomicroscope II with Kodak Ektachrome 160 Tungsten.

Antiricin Antibodies

Ricin was converted to a toxoid by using formaldehyde as described by Olsnes and Pihl (1973). Two New Zealand white rabbits were

subjected to an immunization schedule that began with weekly injections of 50 μg of toxoid and progressively was raised to 250 μg of active toxin. Serum was obtained 20 weeks after the beginning of immunization, and antiricin IgG was purified by ammonium sulfate precipitation and DEAE-cellulose chromatography as described by Rechsteiner (1971).

Iodination of Ferritin

Ferritin (1 mg) was radioiodinated by the chloramine T method as described by Rochsteiner (1971), and ^{125}I was measured on a Beckmann Autogamma 4000 gamma spectrometer.

Materials

The reagents o-dianizidine DiHCl, diaminobenzidine tetrahydrochloride Grade II, peroxidase (horseradish) type II, fluorescein isothiocyanate dextran 70,000 (FITC dextran 70,000; average 64.2 kd), dextran 10,000 (average 9.6 kd), HEPES and ricin (lot 51F-9510) were all obtained from Sigma Chemical. Polyethylene glycol (PEG) 1000 (average 0.95–1.05) was obtained from J. T. Baker Chemical. Sucrose was purchased from Fisher Scientific. Trypan blue, 0.4% in saline was obtained from Grand Island Biological. Ferritin (2× crystallized, cadmium-free) was obtained from Miles Laboratories, and glutaraldehyde was from Polysciences. Lucifer yellow CH was from Aldrich Chemical. Tissue culture supplies were from Flow Laboratories.

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